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Influence of residual bacteria on periapical tissue healing after chemomechanical treatment and root filling of experimentally infected monkey teeth

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The purpose of this study was twofold: first, to determine the influence on the healing of the periapical tissues when selected bacterial strains and combinations thereof remain after root canal treatment; and, second, the relationship to healing of the quality of the root filling. In eight monkeys, 175 root canals, previously infected with combinations of four or five bacterial strains and with radiographically verified apical periodontitis, were endodontically treated, bacteriologically controlled, and permanently obturated. After 2–2.5 yr, the periapical regions were radiographically and histologically examined. Of these teeth, 48 root canals were also examined for bacteria remaining after removal of the root fillings. When bacteria remained after the endodontic treatment, 79% of the root canals showed non-healed periapical lesions, compared with 28% where no bacteria were found. Combinations of residual bacterial species were more frequently related to non-healed lesions than were single strains. When no bacteria remained, healing occurred independently of the quality of the root filling. In contrast, when bacteria remained, there was a greater correlation with non-healing in poor-quality root fillings than in technically well-performed fillings. In root canals where bacteria were found after removal of the root filling, 97% had not healed, compared with 18% for those root canals with no bacteria detected. The present study demonstrates the importance of obtaining a bacteria-free root canal system before permanent root filling in order to achieve optimal healing conditions for the periapical tissues.

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Healing/non-healing in the periapical region after endodontic treatment in humans was first studied radiographically by Strindberg (1). Subsequent reports (2–7) have published radiographic studies of the periapical healing process. They all report a high percentage of failures after endodontic treatment.

In a bacteriological and radiographic study, Engström (8) found a higher healing rate after endodontic treatment when bacteriological samples from the root canal showed no bacteria before root filling. In dogs, histological and/or radiographic studies of the healing process after endodontic treatment have been carried out by a number of investigators (9, 10). A corresponding endodontic study was also performed in monkeys (11). All of these investigations show the impact of residual infection in preventing healing of the periapical tissues. The infecting bacteria were not identified in any of these studies.

The intraradicular flora of teeth with untreated, infected root canals and primary apical periodontitis is known to be different from that of the remaining flora of root-filled teeth associated with apical periodontitis (12–17). Most studies of the intracanal flora of root-filled teeth with non-healed apical periodontitis report the prevalence of enterococci, streptococci, lactobacilli or other Gram-positive rods (12–21). This suggests that some bacterial species may have the ability to survive the antimicrobial and mechanical stages of treatment and establish a persistent root canal infection. However, none of these studies show if the bacteria discovered after removal of root fillings remain from a previous endodontic treatment or if re-infection has occurred. The information currently available regarding the endodontic selection process comes from studies in which the changing composition of the root canal flora has been followed up during various stages of treatment (21–23) and in which the flora present at the time of obturation of the root canals has been identified (24–26). The results from these studies indicate that selection of the microbial species takes place not only during the chemo-mechanical treatment of the canal. Certain species colonizing root canals may also have the advantage of surviving as a result of adaptation to the modified environment caused by the treatment.

The reason why certain bacteria may endure and survive root-filling procedures and materials is unknown. Nevertheless, studies in which the root canals were sampled prior to root filling and where the outcome of treatment of teeth with infected canals was followed up, have shown that some lesions may heal, even when bacteria remain in the canals at the root-filling stage (13, 15–17). To our knowledge, no publication exists that reports on the post-treatment fate of identified microorganisms which persist at the root-filling stage.

In a previous publication (27), we reported on the experimental induction of apical periodontitis in monkeys by inoculation with combinations of well-known bacterial strains and the survival rate of these bacteria after chemo-mechanical treatment of the root canals. It seems now of interest to study the capacity of these combinations of identified 'root canal bacteria' that remain after endodontic treatment to prevent the healing process of the periapical tissues.

The aim of the present investigation was to study, after a follow-up period of 2–2.5 yr: (i) the influence on healing of apical periodontitis of known bacteria that remain after the root-filling procedure; (ii) the influence of selected bacterial combinations on the healing process; and (iii) the influence of the root-filling quality on the healing process.

Material and methods

Animals

The present study is based on the experimental design and materials, previously described in detail (27), which were in accordance with the approval of the Ethical Committee of Göteborg University. In short, eight female monkeys (*Macaca fascicularis*), ≈6 yr of age and with an average weight of 4.6 kg (range 4.1–6.5 kg), were used. The animals were bred and handled as described previously (27).

Experimental procedures

After mechanical devitalization of the pulp, a total of 160 root canals in monkeys 1–6 were originally infected with a 'four-strain combination' comprising the following bacterial species: *Streptococcus anginosus* (formerly *S. milleri*, strain Strep-MC1), *Peptostreptococcus anaerobius* (strain P-MC5), *Prevotella oralis* (formerly *Bacteroides oralis*, strain Bact-MC3), and *Fusobacterium nucleatum* (strain Fus-MC4). In 24 root canals in monkeys 7 and 8, this bacterial combination was supplemented with a strain of *Enterococcus faecalis* (strain Strep MC4) (i.e. a 'five-strain combination') (28). The preparation of the inoculated bacterial suspensions has been described in detail in a previous study (27).

At inoculation, treatment, and sampling, each of the experimental teeth was isolated with a rubber dam and chemically disinfected with 30% hydrogen peroxide followed by 5% iodine tincture. The sterility of the operating field of each tooth was confirmed following inactivation of the iodine with VMG sampling fluid containing 5% sodium thiosulfate (12). A sample of this fluid from each tooth to be treated was absorbed from the surface in a charcoal-impregnated cotton pellet, which was transferred to a 3.2-ml glass bottle containing the semifluid transport medium, VMGA III (12), and in the laboratory inoculated into the semifluid culture medium HCMG-Su1a (12).

After 6–8 months, the apical regions were radiographed and bacteriological samples taken. Of the 184 inoculated teeth, 175 had developed periapical lesions (151 with the four-strain combination and all 24 with the five-strain combination) and were included in this study.

The root canals were treated according to a standardized schedule: mechanically by means of files, and chemically with buffered 1% sodium hypochlorite solution followed by 10% hydrogen peroxide solution, and thereafter again with the hypochlorite solution. The purpose was not to eradicate the bacteria in each of the treated root canals. Bacteriological samples were taken after inactivation of the hypochlorite with 5% sodium thiosulfate; thereafter, sampling fluid VMGA I (12) was introduced into the root canal instead of antimicrobial dressing. During a second session 2 wk later, the root canal treatment was repeated and a second sample was taken. Irrespective of any remaining bacteria, the root canals were root filled with chloropercha Nø and gutta-percha points (29), as previously described (27).

Between treatment sessions the coronal cavities were closed with zinc oxide sulfate sealing cement (30) using a technique that allows bacteriological control of the coronal seal of each root canal (31). Newly sterilized sets of instruments were used at each stage of preparation and sampling from each root canal.

After 2–2.5 yr, the root canal fillings were mechanically and aseptically removed, without addition of any solvent, from 48 randomly selected root canals: 36 root canals originally inoculated with the four-strain combination and 12 root canals originally inoculated with the five-strain combination. From these root canals, two final bacteriological samples ('F-samples') were taken. After the first of these samples was taken, the root canal was filled with sampling fluid, a charcoaled paper point was introduced, and the canal was tightly sealed. The second sample was taken 2 wk later, immediately before killing the animals. When one or both of the two F-samples showed growth, the root canal was designated as harbouring residual bacteria and the strains present were identified and recorded. All samples were taken using the pumping and maximal removal of fluid (PMR) method (12).

The animals were given an overdose of penthotal sodium (Pentothal; Abbot, Campo-verde, Italy) and perfused with physiological saline followed by 4% buffered formalin. The jaws were dissected out and cut into blocks containing two or three teeth (including the periapical tissues). The blocks were transferred to, and stored in, bottles containing 4% buffered formalin.

Radiographic examination

The apical regions of all experimental teeth were radiographed every 4–6 months during the observation period.

After the animals were killed, the entire jaws, and thereafter the sectioned blocks, were radiographed in mesio-distal and bucco-lingual directions until sharp radiographs were obtained. The radiographs were independently analyzed by three endodontists. In a few cases where slightly discordant observations were recorded, the result was discussed and re-evaluated, and full agreement was obtained. A regeneration of the periapical space was regarded as a healed lesion (32).

Histological examination

A diamond saw was used to section the jaw into bone blocks containing between one and three roots each. The bone blocks were fixed in 4% neutral formalin, decalcified in 5% formic acid, and embedded in paraffin. Serial sections were made vertically through the apical foramen area of each root to be examined and then stained with hematoxylin and eosin. The inflammation was graded as 'none', 'mild', 'moderate', or 'heavy', according to the intensity of the inflammatory infiltrate. The type of inflammatory reaction was also classified as 'chronic', 'acute', or 'mixed'. Healed periapical tissues were characterized by a lack of inflammation and by restitution of the periodontal membrane. Histological analysis was performed without knowledge of the results of the radiographical examinations. Owing to a failure in processing tissue blocks, histological assessment was not possible for 10 teeth. For these teeth, healing/non-healing was judged by means of radiography alone. The assessment of healing of the remaining 165 teeth was determined by both histological and radiographic examinations. Full agreement between the two examinations was obtained in 154 (93%) of these teeth. In those 11 cases where discrepancies occurred, the histological findings showed non-healed lesions and these were chosen as the deciding factor.

Registration of the root-filling quality

The root fillings were analyzed by means of radiographic and histological examinations and were grouped into four categories in regard to the filling quality: < 0.5 mm from apex; 0.5–2.5 mm from apex; > 2.5 mm from apex (including inadequately sealed root fillings); and overfilled. All four groups were represented in each animal. Full agreement between the observers was obtained regarding placement in the four groups.

Statistical methods

In the statistical determinations of the ratio of non-healed/healed lesions in relation to the remaining bacteria and the quality of the root fillings, the chi-square method with Yates' correction was used. The individual healing capacity of each animal was calculated using Fisher's exact test with the animal as the computational unit. A P-value of < 0.05 was considered statistically significant.

Results

The total frequency of non-healed periapical lesions in relation to the presence or absence of bacteria in the root canals of the eight monkeys is shown in Fig. 1. Altogether, samples from 90 of the 175 root canals showed bacteria at the root-filling stage (Rf samples), while in samples from the remaining 85 root canals, no bacteria were found. In the 90 canals containing bacteria, 71 (79%) did not heal, while in the 85 canals without bacteria, 24 (28%) did not heal. The relationship between the presence/absence of bacteria and healed/non-healed periapical lesions in each animal followed a similar pattern (Table 1). Despite small differences between the animals, the observation that the presence of bacteria correlates with non-healed periapical lesions showed statistical significance ($P < 0.01$), using the animal as a computational unit.

In 48 root canals (Table 2), samples were also taken after removal of the root filling (F samples). Thirty-one of these canals contained bacteria; of these, 30 (97%) had not healed. The 17 of the periapical lesions at root canals with no bacteria in the F samples had healed; of these, 11 had previously shown negative Rf samples. This difference between the number of non-healed and healed lesions in the presence and absence of bacteria is statistically significant ($P < 0.01$).

Table 3 shows that when the periapical lesions containing bacteria of the four-strain combination (monkeys 1–6) did not heal, no discernable pattern of bacterial combinations was found among the different monkeys (monkeys 1–6).

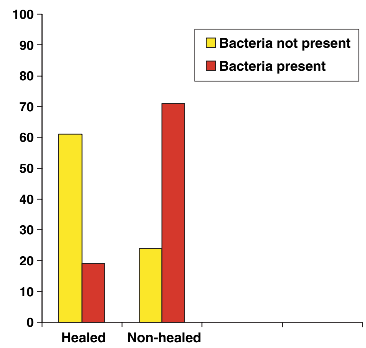

Fig. 1. Number of healed and non-healed periapical lesions in relation to root canals, with ($n = 90$) and without ($n = 85$) bacteria persisting 2–2.5 yr after root filling.

Table 1

Frequency of bacteria in root-filling stage (Rf samples) (n = 175) of each monkey

	Bacteria present ($n = 90$)		No bacteria present ($n = 85$)		
Monkey	Non-healed	Healed	Non-healed	Healed	Total
1	3	4	4	10	21
2	17	3	1	4	25
3	7	1	4	13	25
4	3	5	2	18	28
5	13	2	5	7	27
6	12	2	6	5	25
7	7	1	1	3	12
8	9	1	1	1	12
Total	71	19	24	61	175

*Non-healed ($n = 95$) and healed ($n = 80$) periapical lesions after 2–2.5 yr, with and without bacterial infection.

Table 2

The frequency of surviving bacteria in root-filling stage samples (Rf samples) and after removal of the root filling (F samples) from 48 root canals in relation to non-healed and healed periapical lesions

	Number of roots containing bacteria		Number of lesions	
Bacterial findings	Rf samples	F samples	Non -healed	Healed
Anaerobic and facultative bacterial strains	29	26	26	0
Facultative bacterial strains only	8	5	4	1
No re-isolated bacterial strains	11	17	3	14
Total	48	48	33	15

Anaerobic bacterial strains = one or more of the anaerobic strains in the four- or five-strain combinations.
Facultative bacterial strains = *Streptococcus anginosus* and/or *Enterococcus faecalis*.

In those root canals where the lesions healed despite residual bacteria, the predominant strain was *S. anginosus*, and in 32 root canals containing only this strain, 15 had healed. In contrast, where combinations of anaerobic bacteria and *S. anginosus* remained, only one lesion out of 40 had healed. On the other hand, in monkeys 7 and 8 (containing the five-strain combination, Table 4), 12 out of 16 Rf samples from roots with non-healed periapical lesions retained the complete combination in the root canals. In each of these two monkeys, only one root canal among those that showed healed lesions contained bacteria.

Figure 2 shows the frequency of non-healed periapical lesions in 95 root canals and the presence or absence of bacteria in relation to the root-filling quality. The number of non-healed periapical lesions was equally distributed in three of the four quality groups (< 0.5 mm and 0.5–2.5 mm from the apex and overfilled) with 23, 25, and 20 lesions, respectively; in total, 68 out of the 71 root canals with bacteria present. A similar distribution between the quality groups was observed in the 24 root canals without bacteria. In the group > 2.5 mm from the apex five lesions had not healed; three of these contained bacteria.

Figure 3 shows the frequency of the presence or absence of bacteria in the 80 totally healed periapical lesions, in relation to the quality of the root filling. Sixty-one root canals contained no bacteria; 28 of these had a well-performed root filling. Only three canals in this healed group had bacteria in the Rf samples. When the periapical lesions healed in spite of bacteria present in the Rf samples, the quality groups < 0.5 mm from the apex and overfilled dominated. The sample number of root canals in each of these categories in each monkey was too small to allow statistical calculations to be performed.

Discussion

The negative impact of bacteria on the outcome of root-canal treatment and root filling has been shown in earlier clinical cross-sectional studies in humans (13–17, 19, 33–35). However, for obvious ethical reasons, no prospective follow-up study has been performed on this issue in humans. Even if the finding, that bacteria persisting in the root canal have a serious impact on the periapical healing process in permanently root-filled teeth, is not new, the fate of bacteria that are left behind during the root-filling process has been largely unknown.

The purpose of the present study was to determine whether some selected bacterial strains, persisting after

Table 3

The frequency of bacterial strains present in the root-filling stage samples (Rf samples) (n = 72) of monkeys 1–6 (infected with the four-strain combination) in relation to non-healed and healed periapical lesions at the end of the observation period (2–2.5 yr)

	Bacteria present in Rf samples											
	Non-healed (*n* = 55) Monkey number						Healed (*n* = 17) Monkey number					
Bacterial strains	1	2	3	4	5	6	1	2	3	4	5	6
S. anginosus + *Pept. anaerobius* + *Prev. oralis* + *F. nucleatum* (the inoculated combination)		1	4		1							
S. anginosus + *Pept. anaerobius* + *Prev. oralis*						1						
S. anginosus + *Pept. anaerobius* + *F. nucleatum*		3	1		2							
S. anginosus + *Prev. oralis* + *F. nucleatum*		1		1								
S. anginosus + *Pept. anaerobius*		2			4	1						
S. anginosus + *Prev. oralis*	1				1	1						
S. anginosus + *F. nucleatum*	2	3				2				1		
Prev. oralis + *F. nucleatum*				1								
Pept. anaerobius		2			1	1						
F. nucleatum					1		1					
S. anginosus	0	5	2	1	3	6	3	3	1	4	2	2
Total number of root canals with bacterial presence (*n* = 72)	3	17	7	3	13	12	4	3	1	5	2	2

Table 4

The frequency of bacterial strains present in the root-filling stage samples (Rf samples) (n = 18) of monkeys 7 and 8 (infected with the five-strain combination) in relation to non-healed and healed periapical lesions at the end of the observation period (2–2.5 yr)

	Bacteria present in RF samples			
	Non-healed ($n = 16$)		Healed ($n = 2$)	
Bacterial strains	Monkey 7	Monkey 8	Monkey 7	Monkey 8
S. anginosus + *Pept. anaerobius* + *Prev. oralis* + *F. nucleatum* + *E. faecalis* (the inoculated combination)	6	6		1
S. anginosus + *Pept. anaerobius* + *Prev. oralis* + *E. faecalis*		1		
S. anginosus + *Prev. oralis* + *E. faecalis*		1		
E. faecalis	1	1	1	
Total number of root canals with bacterial infection ($n = 18$)	7	9	1	1

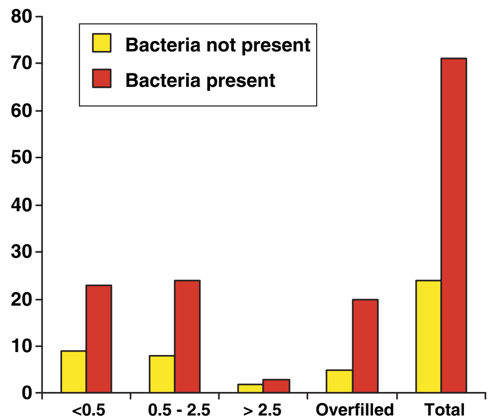

Fig. 2. Number of non-healed periapical lesions ($n = 95$) in relation to persisting infection and the four groups of root-filling quality: < 0.5 mm, 0.5–2.5 mm, and > 2.5 mm from the apex (including inadequately sealed), and overfilled.

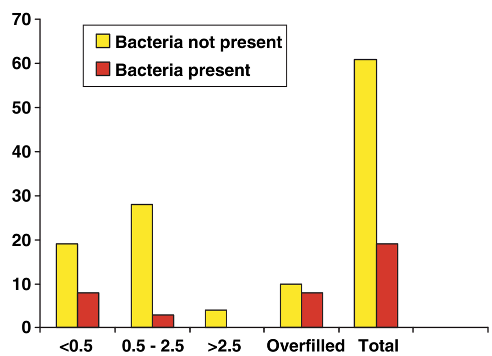

Fig. 3. Number of healed periapical lesions ($n = 80$) in relation to persisting infection and the four groups of root-filling quality: < 0.5 mm, 0.5–2.5 mm, and > 2.5 mm from the apex (including inadequately sealed), and overfilled.

the root-filling treatment, could survive in root-filled canals and prevent periapical healing. The study was based upon a previous report (27) in which infections were experimentally established and periapical lesions were induced by known bacterial combinations. The teeth were endodontically treated by chemo-mechanical means, permanently root filled, and followed up radiographically and histologically at the end of the observation period of 2–2.5 yr. Seventy-nine per cent of teeth with root canals containing bacteria at the root-filling stage failed to heal, while teeth with root canals without bacteria had periapical lesions in 28% of cases. In samples from the root canals of 48 randomly selected teeth, bacteria were identified after removal of the root fillings (F samples) at the end of the follow-up period (Table 2). Inoculated bacteria, or combinations thereof, were found in 31 (82%) of the 38 root canals that contained bacteria in the Rf samples. Of these, 97% had not healed. All these findings demonstrate the significant impact ($P = 0.01$) of bacterial presence on the post-treatment outcome and support earlier cross-sectional studies (12–21) showing that bacteria can survive in root-filled canals for many years and be an important cause of non-healing of periapical tissues.

It has been presumed that the occurrence of apical periodontitis may be the result of certain factors, such as later infections caused by coronal leakage. No bacterial strains, other than those originally inoculated, were isolated in any sample, indicating that no coronal leakage had occurred. In the root canals with bacteria present when the root filling was removed, 30 of the 31 canals had persisting periapical lesions. This indicates strongly that those bacteria present at the root-filling stage not only survived within the root canal for more than 2 yr, but also have the capacity to maintain the periapical inflammation and prevent the healing process.

It could be argued that the main result of this study could be falsely interpreted as a result of variations in the healing/non-healing response between the animals. The statistical outcome was therefore calculated using the animal as the computational unit. The correlation between positive bacterial samples and non-healed periapical conditions on the one hand, and negative bacterial samples and healed periapical lesions on the other, can, in spite of some individual differences, be considered conclusive ($P = 0.01$).

The rate of failed healing in the infected teeth (79%) may seem high, given the argument that a technically well-performed root-filling would seal the root canal and embed the bacteria inside the canal without communication with the periapical tissues. This would be quite plausible, if bacteria were present only in the main canal (e.g. the smear layer) (36, 37) or in the dentinal tubuli (38). If that is the case, healing of the periapical tissues can occur in spite of remaining bacteria. Even if it has been found to be impossible to eliminate dentine contamination using root canal debridement and irrigation alone (39), it is obvious that bacteria in that location have little influence on the periapical tissues (40). However, bacteria can penetrate into the root canal periphery (e.g. the apical delta and side canals), which are difficult to reach not only by chemo-mechanical procedures but also with the permanent root filling, and the result can be longstanding endodontic infections (41). Even if the harsh conditions lead to a selection process with 'stressed' living conditions for the bacteria, some may survive for longer periods of time after the formation of biofilm communities, and the chronic inflammatory process in the periapical tissues is thus maintained (21). This hypothesis is the most plausible explanation for the correlation between the presence of bacteria in root-filled teeth and chronic apical periodontitis.

In the present study, the root fillings were analyzed radiographically and histologically and grouped into four categories with regard to the root-filling quality (< 0.5 mm, 0.5–2.5 mm, or > 2.5 mm from the apex, or inadequately sealed; and overfilled). All four groups were represented in each animal and there were no significant differences either on an individual, or on a tooth-based, level between the groups with respect to the distribution of teeth with non-healed periapical lesions, with or without bacteria (Fig. 2). Similarly, healing of periapical lesions occurred in all four groups (Fig. 3). This study confirms numerous clinical evaluation studies (1, 2, 18, 41–48), which have shown a correlation between a technically inadequate root filling and apical periodontitis. However, a permanent root filling would have no decisive effect on the outcome, even if technically well performed. In contrast, if bacteria are not present at the root-filling stage, one could well foresee healing of the apical periodontitis, as the quality of the root filling was found to be of less importance for the healing process than the presence or absence of bacteria.

Fifteen of the 17 bacteria-containing root canals primarily infected with the four-strain combination that showed healed periapical lesions contained only *S. anginosus* and no anaerobes. This indicates that when only streptococci remain, they may be less capable of maintaining a periapical lesion compared with polymicrobial infections (Table 3). It is interesting to note that the addition of *E. faecalis* to the collection (five-strain combination, Table 4) resulted in higher survival of the complete combination, than the same bacterial combination without *E. faecalis* (four-strain combination, Table 3). This also illustrates the importance of bacterial synergism in polymicrobial chronic root canal infections, as shown in numerous acute experimental infections (27, 28, 49–55).

In 85 root canals no bacteria were found at the root-filling stage. In spite of this, the periapical lesions did not heal during the observation period in 24 cases (28%). A number of explanations for this could be suggested. Bacteria may have been missed as a result of sampling or laboratory failures (false-negative samples). However, there was good agreement between the Rf- and F samples from 48 root canals (Table 2), indicating that the number of false-negative samples was low with the sampling technique used (12). A non-healed lesion might also depend on a tissue response to factors other than bacteria. Yet, it has been shown that necrotic tissue in itself does not cause an inflammatory periapical reaction (31). The inflammatory reaction may also remain owing to a reaction against the root-filling material, although the gutta-percha used has been documented as being harmless to the tissue (29). This was confirmed in the present study by the fact that overfilled canals gave more healed lesions than underfilled canals. Another explanation may be that the bone healing process after endodontic treatment can be surprisingly slow (1, 56, 57) and a longer observation period may be needed for complete bone regeneration in some cases.

The overall success rate in the present study (71%) may seem low in comparison to human clinical studies in which healing of apical periodontitis has been reported to occur in 85–90% of cases after endodontic treatment (1, 42, 56, 58, 59). It should be pointed out that the purpose of this study was not to necessarily eradicate all bacteria from every root canal, but to perform a regular and controlled, somewhat standardized, chemo-mechanical treatment. In order to fulfill the experimental purpose, sampling fluid was used in the root canals instead of antibacterial interappointment dressing, and it has been shown by several investigators (6, 7, 9, 10, 22, 38, 39) that interappointment dressings are important for eradicating bacteria in root canals. A contributing factor to the relatively small number of healed cases may also be the fact that healing/non-healing was judged on the basis of histology. If only radiographs had been used, 11 more healed lesions would have been registered.

In conclusion, this study has shown that bacteria can survive a permanent root filling for several years and that the presence of bacteria significantly correlates with non-healed apical periodontitis. Combinations of remaining bacterial strains were more frequently related to non-healed lesions than were single strains. A permanent root filling has limited effect on the outcome, even if it is technically well performed. If bacteria are eliminated at the root-filling stage, the prognosis for healing is good.

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